A NEW DIFFERENTIAL CULTURE MEDIUM FOR THE CHOLERA VIBRIO *

PLATE 21

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After it was found in this laboratory that a combination of eosin and methylene blue in certain definite proportions in lactose agar yielded an excellent plate medium for differentiating colonies of B. typhosus from those of B. coli,[1] experiments were planned to determine whether a similar differential medium for the cholera vibrio could be prepared. The cholera vibrio grew well on nutrient agar containing as much as 0.1% of eosin; but methylene blue and a number of other basic stains were found to inhibit the growth of the cholera vibrio to such an extent that their use for our purpose seemed out of the question. When the basic stains were used in combination with eosin, their toxicity for the cholera vibrio was much reduced, but not sufficiently to allow of their use in a strength approaching that in use in the typhoid medium.

However, bismarck brown proved to be far less toxic for the cholera vibrio than the other basic stains that we had previously used and hence our efforts were concentrated on it. The cholera colony was somewhat different in color from the colony of B. coli on nutrient agar containing eosin alone after 48 hours' incubation; the former had a red center, whereas the latter was uniformly pink. When a small amount of nutrose was added to the culture medium, the colonies were larger and this difference in color became apparent in 24 hours. Parallel tests were then carried out with yellowish eosin, bluish eosin, the pure French eosin, eosin A.G., and with erythrosin. The bluish eosin gave decidedly better differentiation than did the other stains.

On sugar-free nutrient agar to which were added 1% saccharose, 0.25% nutrose, and 0.0625% bluish eosin, the cholera colonies had red centers, while the colonies of B. coli were uniformly pink. We planted many mixtures of normal stools and cholera cultures on this medium and after 24 hours' incubation the cholera colonies could

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[1] Jour. Infect. Dis., 1916, 18, p. 596.

be distinguished at a glance from the other colonies, because the centers of the cholera colonies were several shades deeper in color than the other colonies and because the latter were of approximately the same depth of color at the center as near the periphery.

Bismarck brown was employed in combination with all the different eosins mentioned, and here, too, the bluish eosin yielded better results than the other eosins. Various proportions of the two stains were used, 0.0625% of eosin and 0.04% bismarck brown being finally selected as the optimum. The two strains added to nutrose saccharose agar in these proportions gave a medium on which the cholera colonies showed dark brown centers in 24 hours, while the colonies of B. coli were of a pale-pinkish or yellowish color (see Plate 21). The differentiation of the cholera colony was therefore much more striking than it was in the medium containing only eosin. When the cholera colony was buried beneath a large colon colony it might still be apparent as a minute black dot; on staining a smear from this dark area of the colony of B. coli, well-curved vibrios were seen. By transferring such material to peptone solution the cholera colony could readily be isolated from such a mixed colony in pure culture.

We prepare our medium in the following manner: Two pounds of chopped beef are soaked in 2 liters of distilled water in the ice-box overnight. The fluid is squeezed out, heated in the Arnold sterilizer, filtered through filter paper, made neutral to litmus by the addition of sodium hydrate solution, and again heated. After being allowed to cool it is inoculated with B. coli and incubated for 2 or 3 days. Nutrient agar is then prepared from it by adding 1% Witte's peptone, and 0.5% sodium chlorid, and clearing with egg in the usual way. The reaction is adjusted to —0.5. The nutrose (0.25%) is added after the agar has been cleared and filtered. A stock aqueous solution of bluish eosin (3%) is kept on hand in the dark. A 1% solution of bismarck brown in water containing 10% alcohol is also kept in stock. The bismarck brown is not completely soluble to 1% in distilled water alone.

To 50 c.c. of the nutrose agar are added 1% saccharose, 1 c.c. of 3% eosin solution, and 2 c.c. of 1% bismarck-brown solution. After this mixture has been shaken until the stains are uniformly distributed throughout the agar, plates are poured. Before being inoculated, the plates are uncovered and placed face down on the shelf of the incubator for 20 or 30 minutes to remove any excess of moisture.

Agar prepared with Liebig's meat extract, instead of the meat infusion rendered sugar-free by B. coli, yields similar results, but the differentiation of the vibrio colonies from the other colonies is not so good.

Usually we do not heat the medium after the stains have been added, but we have shown that such heating has no deleterious effect upon it.

When eosin and bismarck brown are added to distilled water in the proportions just mentioned, precipitation takes place; this never occurs however in the presence of the agar, the latter obviously acting as a "Schutzkolloid."

The dark center of the colony is due for the most part to the staining of the vibrios themselves, tho the medium immediately beneath the center of the colony is seen to be dark if the colony is scraped away.

Old laboratory strains of cholera differ greatly from freshly isolated ones in the size and appearance of their colonies on plain nutrient agar, in the degree of motility, in the rapidity of fermentation of sugars, and in a number of other respects. The results that we have obtained cannot be duplicated satisfactorily with old laboratory strains.

Our own strains have necessarily been grown on artificial culture media for several months; it is possible that strains fresh from cholera feces would give the same picture on bismarck-brown eosin agar without nutrose that our present strains yield on this medium with nutrose.

Through the kindness of Dr. Otto Schöbl we were in a position to plant 18 cholera-like vibrios on our medium. These vibrios had been isolated by him from cholera suspects or cholera contacts and resembled the cholera vibrio in almost all particulars, but they failed to agglutinate with anticholera serum. They all had a single flagellum, gave a positive indol reaction, and were hemolytic. Their colonies were found to resemble those of the true cholera vibrio very closely on our medium. This is perhaps an advantage rather than a disadvantage; for if cholera-like vibrios have been seen in the peptone it is well to be able to find them on the plates and show that they are not cholera vibrios.

We planted a large number of stools on Endo plates containing saccharose instead of lactose and then transplanted the red colonies

developing on these plates to agar slants. The saccharose-fermenting organisms obtained in this manner were later inoculated on the bismarck-brown eosin plate and in no instance did colonies resembling those of the cholera vibrio occur.

This medium will have to be tried out in the isolation of vibrios from actual cases of cholera and from convalescents from cholera in order to determine whether or not it possesses practical value in the diagnosis of cholera. It will probably prove to be much superior to the plain agar plate but decidedly inferior to the Dieudonné medium. In combination with a strongly alkaline peptone solution it may yield excellent results.

After this work was completed Aronson[2] described a special medium for the isolation of the cholera vibrio. It is prepared as follows: 35 gm. of agar are added to 1 liter of hydrant water and the whole allowed to stand overnight. After the addition of 10 gm. of meat extract, 10 gm. of Witte's peptone, and 5 gm. of sodium chlorid, the mass is heated in a steam sterilizer at 100 C. for from 4 to 5 hours. The flask containing the hot agar is then slanted to allow the coarser particles to settle out and then the agar is poured into flasks graduated to receive 100 c.c. each.

The following stock solutions are prepared and heated one-half hour in the steam sterilizer: (a) 10% solution of dry sodium carbonate, (b) 20% solution of cane sugar, (c) 20% solution of dextrin. A saturated alcoholic solution of basic fuchsin and a 10% solution of sodium sulfite, the latter sterilized by being brought to the boiling point, are also kept in stock.

To 100 c.c. of the agar are added 6 c.c. of the 10% sodium carbonate solution, and the containing flask is heated 15 minutes at 100 C. The agar takes on a dark-brown color and becomes very cloudy. While the agar is still hot, the following are added: 5 c.c. of the 20% cane-sugar solution, 5 c.c. of the 20% dextrin solution, 0.4 c.c. of the alcoholic-fuchsin solution, and 2 c.c. of the 10% sodium-sulfite solution. The flask is held in a slanting position until the coarser particles settle to the bottom; then plates are poured. The last of the agar, containing the sediment, is discarded.

Cholera strains should yield large red colonies on this medium in from 15 to 20 hours; at this time the colonies of B. coli are small and colorless.

[2] Deutsch. med. Wchnschr., 1915, 42, p. 1027.

We prepared Aronson's medium according to these directions, but our strain of cholera which had now grown for several months on artificial culture medium, did not yield red colonies promptly. After adding 0.25% nutrose to the medium, however, we obtained remarkably good results with mixtures of our culture and normal stools.

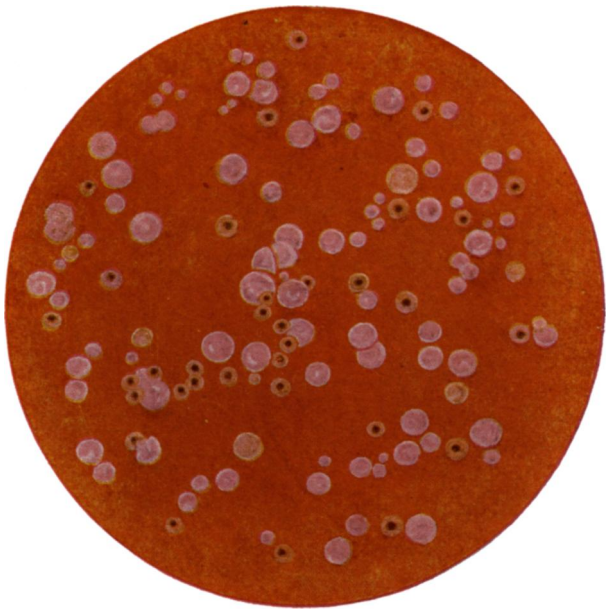
We then substituted in this medium eosin and bismarck brown in the proportions recommended here for the fuchsin and sodium sulfite; similar results were obtained. The differentiation of the cholera colonies is as striking as on the eosin bismarck-brown agar of reaction —0.5 and far greater amounts of feces can be safely inoculated on the strongly alkaline plates.

The colonies of B. coli on the Aronson medium become red on further incubation, obscuring the cholera colonies. On the eosin bismarck-brown strongly alkaline agar the colonies of B. coli remain pale after 48 hours' incubation and the cholera colonies are much darker than after 24 hours' incubation; hence the cholera colonies, instead of becoming obscured, are even more sharply differentiated from the other colonies upon further incubation.

Finally, we substituted for the fuchsin and sodium sulfite in the Aronson medium plus nutrose, 4 c.c. of 3% bluish eosin and obtained excellent results with this medium also. The cholera colonies were colored deep-red while the colonies of B. coli were pink.

Aronson's medium and our two modifications of it give results very similar to what one is accustomed to get with the Dieudonné medium. Which of these four kinds of media is best can only be determined by extensive parallel tests on the stools of convalescents from cholera. The first three media possess the advantage over the Dieudonné medium of being always ready for immediate use.

Plate 21



Reproduction of a bismarck-brown eosin plate inoculated with a mixture of a normal stool and cholera vibrios. The cholera colonies have dark-brown centers.